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ATOMIC FORCE MICROSCOPY STUDY OF FINE STRUCTURES OF THE ENTIRE SURFACE OF RED BLOOD CELLS

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Abstract

Glutaraldehyde-fixed red blood cells were imaged by tapping mode atomic force microscopy (TMAFM) in air at room temperature. The results show that TMAFM can visualize the morphology of the red blood cell at both cellular and nanometer scales. The scan size covers the range from several hundred nanometers to more than one hundred micrometers. TMAFM not only has a higher resolution than the optical microscope, but also can observe biological samples without precoating as required for scanning electron microscopy (SEM). The AFM images of the entire surface of an uncoated red blood cell with nanometer resolution are successfully reconstructed by 28 AFM images of the preselected sub-areas on the surface of the red blood cell. These images reveal directly the fine structures of the external surface of uncoated red blood cells in air. The surface exhibits a characteristic structure composed of a large number of closely-packed nanometer particles with a size ranging from a few nanometers to tens of nanometers. These "particulate" components are evenly distributed, and no jumping protrusion or depression structures were found. These particles give rise to a very smooth surface of the red blood cell as shown in a large-scan AFM image. In addition, the 28 AFM images obtained by the continuous scanning over 3 hours indicate that TMAFM can image soft biological samples such as red blood cells stably and reproducibly.

Key Words: Atomic force microscopy, red blood cell, surface structure.

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Introduction

The invention of the scanning tunneling microscope (STM) has led to a revolution in surface science due to the extremely high resolution of this microscope. Based on STM, several scanning probe microscopes (SPMs) have been developed in the past few years, such as the atomic force microscope (AFM), lateral force microscope (LFM), magnetic force microscope (MFM) and photo scanning tunneling microscope (PSTM). Among these microscopes, AFM has attracted most attention in biological applications because it can image both conducting and non-conducting materials. AFM has been used to image structures of biological samples, such as nucleic acids (Bezanilla *et al.*, 1992; Hansma *et al.*, 1992, 1994; Thundat *et al.*, 1992a, 1992b; Vesenka *et al.*, 1992, 1993; Zhang *et al.*, 1994), proteins (Karrasch *et al.*, 1994; Yang *et al.*, 1994), and cells (Henderson *et al.*, 1992; Hörber *et al.*, 1992). In the earlier studies, AFM has already be used to examine the surface of red blood cells (RBC). Studies on the morphology of red blood cells in air or in solution have been carried out by contact mode AFM (Butt *et al.*, 1990; Gould *et al.*, 1990; Häberle *et al.*, 1991; Zachee *et al.*, 1994), but the integral morphology of the whole surface of red blood cell at a high resolution has not yet been reported. In other words, we do not have complete knowledge of the whole red blood cell at the nanometer scale.

Although scanning electron microscopy (SEM) can be used to investigate the morphology of a variety of biological samples at the tissue, cell, and biomacromolecular level, the samples often require special treatment, such as coating, staining, and dehydration and drying in vacuum. Sometimes, more complicated techniques, e.g., freeze-fracture replication, are necessary for the study of surface structures. Surface information is thus obtained in an indirect manner and carries a risk of producing artifacts.

In comparison with conventional SEM techniques, AFM has the following advantages: (1) the sample preparation procedures are simple and convenient; staining and coating is not necessary; (2) since one can observe

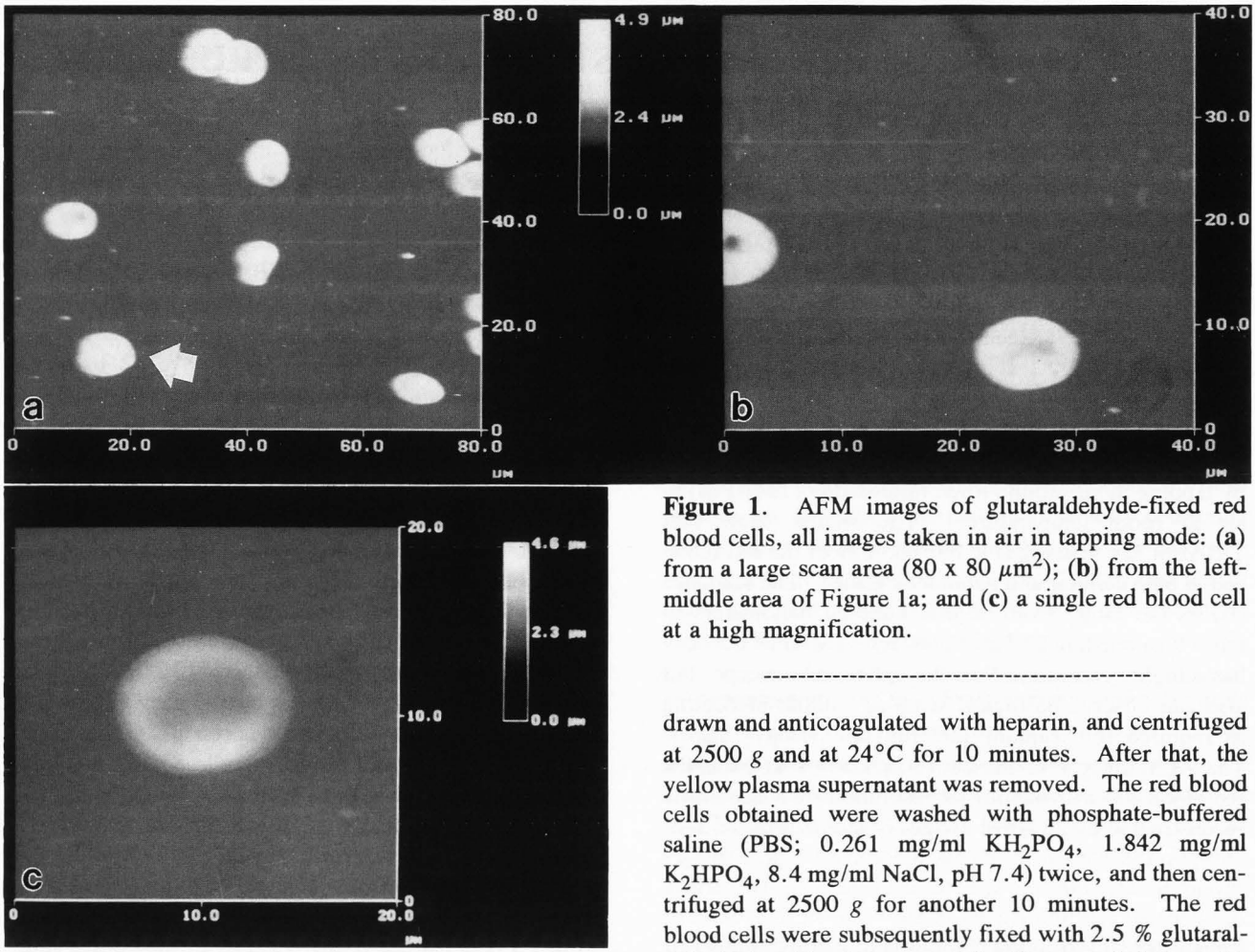

Figure 1. AFM images of glutaraldehyde-fixed red blood cells, all images taken in air in tapping mode: (**a**) from a large scan area (80 x 80 μm^2); (**b**) from the left-middle area of Figure 1a; and (**c**) a single red blood cell at a high magnification.

samples in AFM in air or in liquid, drying in vacuum is not required for sample preparation. Nevertheless, because of the strong interactions between the tip and the sample surface, contact mode AFM often leads to surface damage, movement, or even complete destruction of the samples during scanning. Therefore, contact-mode AFM does not easily produce stable and reproducible images of biological samples, especially for soft ones or those not well anchored on the substrate. Fortunately, tapping mode AFM, which was developed in recent years is able to reduce the lateral interactive force during scanning and so permit prolonged scanning of soft surfaces. In the present study, we image the morphology of the surface of uncoated red blood cells with TMAFM from cellular to nanometer scale. The nanometer-scale fine structures of the whole surface of an uncoated red blood cell is studied in detail. In addition, stability issues related to the AFM of imaging samples of red blood cells are also discussed.

Material and Methods

Preparation of red blood cells

Venous blood from healthy human volunteers was drawn and anticoagulated with heparin, and centrifuged at 2500 *g* and at 24°C for 10 minutes. After that, the yellow plasma supernatant was removed. The red blood cells obtained were washed with phosphate-buffered saline (PBS; 0.261 mg/ml KH_2PO_4, 1.842 mg/ml K_2HPO_4, 8.4 mg/ml NaCl, pH 7.4) twice, and then centrifuged at 2500 *g* for another 10 minutes. The red blood cells were subsequently fixed with 2.5 % glutaraldehyde for 30 minutes, and finally washed thrice with PBS. All of the above procedures were completed within one hour after the blood was drawn. Four μl of this suspension of fixed red blood cells was added onto the freshly-cleaved mica surface and left to adsorb for about 20 seconds. Then, the residual solution was removed with a piece of filter paper. After that, the red blood cells deposited on the mica surface were washed with distilled water thrice to avoid possible contamination caused by salt crystals. After air-drying, the sample was imaged by AFM in tapping mode under ambient conditions within one day.

Experimental instruments

The AFM experiments on the red blood cells were performed on a NanoScope III SPM (Digital Instruments Inc., Santa Barbara, CA). The height (z) and lateral measurement (x, y) of the stage were calibrated with an 180-nm step height standard and a diffraction grating, respectively. Commercially available tapping mode cantilevers were used (Digital Instruments). The cantilever was 125 μm long with a resonance frequency of about 313 kHz. A freshly-cleaved mica surface was used as substrate for deposition of the specimens of the

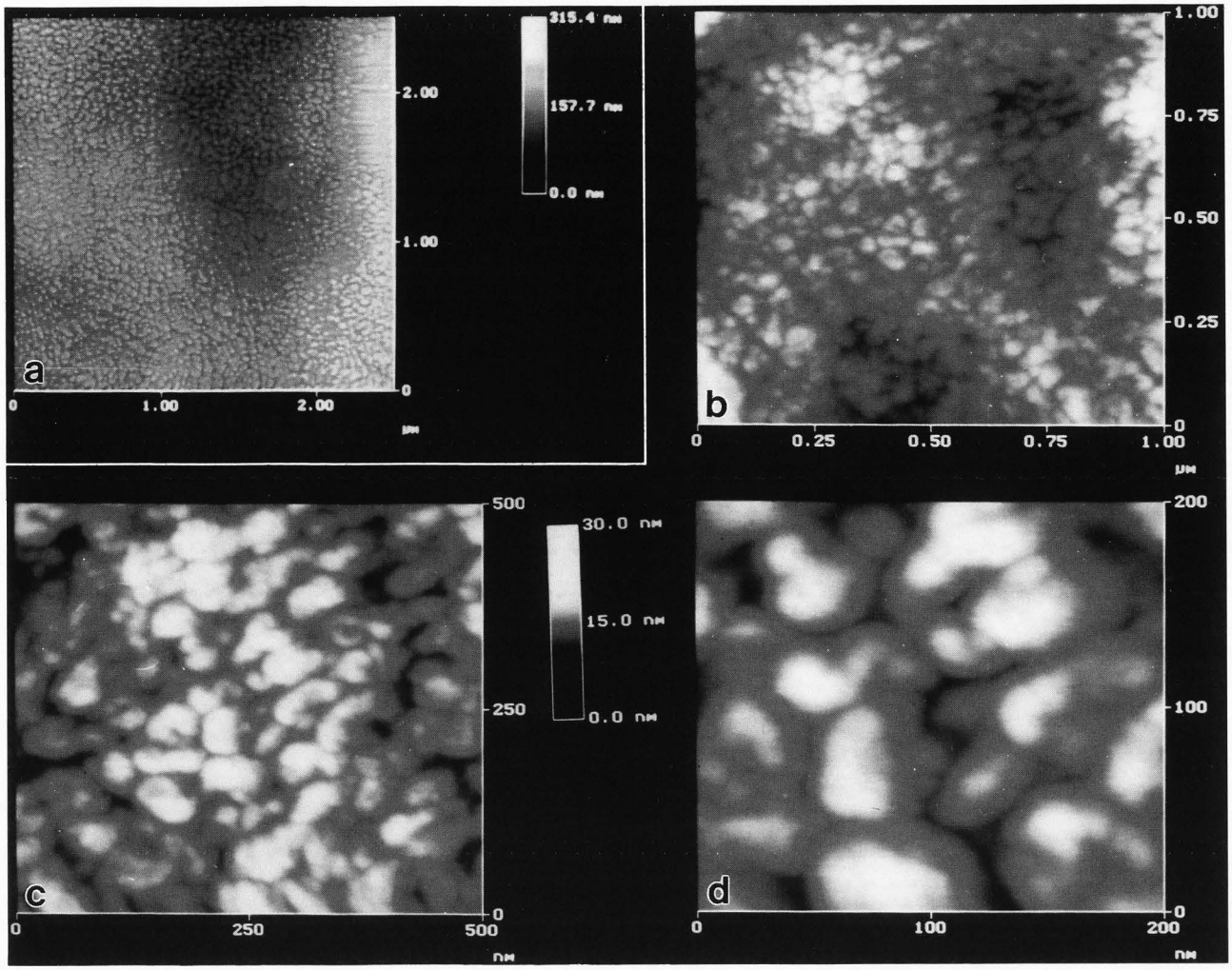

Figure 2. AFM images of the fine structure of local areas on the surface of red blood cells at different magnifications. Scan-sizes: (**a**) 2.5 x 2.5 μm^2; (**b**) 1.0 x 1.0 μm^2; (**c**) 500 x 500 nm^2; and (**d**) 200 x 200 nm^2. The intensity bar in upper row refers to Figure 2a; the lower intensity bar referes to Figures 2b-2d.

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red blood cells. The fixed red blood cells were imaged on the AFM in tapping mode under ambient conditions within one day.

Results and Discussion

Visualization of red blood cells at the cellular scale

Large scan TMAFM images of fixed red blood cells on mica are shown in Figures 1a, 1b and 1c. These images were stable and reproducible during repeated scanning. As observed in a conventional SEM, the red blood cells have a tendency to stick to each other or form stacks. In the top-middle area of Figure 1a, two overlapping red blood cells can be seen. Section analysis shows that those red blood cells (e.g., marked by a white arrow in Fig. 1a) actually form a stack of two cells since their thickness is approximately twice that of a single red blood cell. In other areas, the cells (Fig. 1c) are scattered individually. A low cell concentration favors and allows imaging of a single red blood cell. Stable and reproducible AFM images of the red blood cells can usually be obtained by tapping mode because the accumulation of lateral force exerted by the TMAFM tip on the surface of the red blood cell was greatly reduced. However, sudden vibration during scanning should be avoided in order to eliminate transient strong interactions between tip and sample; sudden vibration sometimes may cause sample damage and degrade instrumental stability. The experiment cannot be continued after the apex of AFM tip has picked up debris from the damaged surface of red blood cells.

In Figure 1a, most of the red blood cells appear doughnut-shaped. Their diameters are around 7.2 μm and their thickness about 1.0 μm. The size and shape of

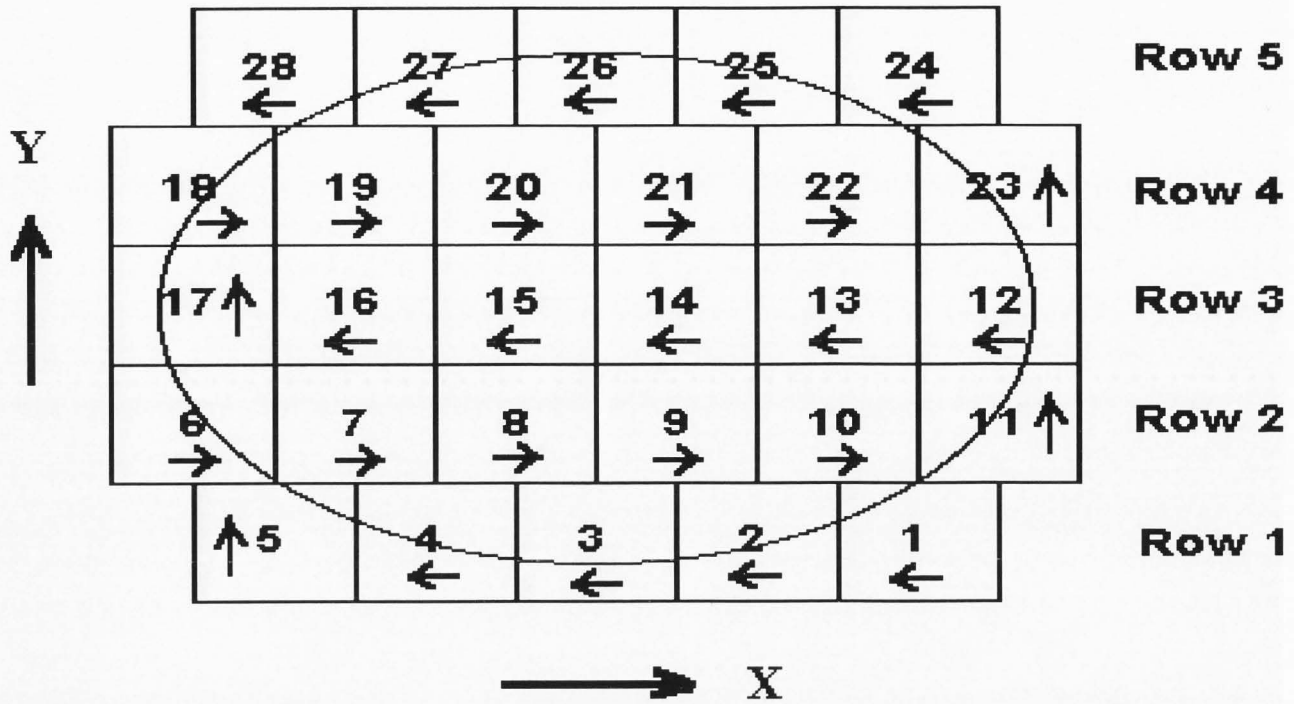

Figure 3. Schematic diagram of the process of sequentially recording 28 AFM sub-area images of the entire surface of a red blood cell.

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the central depression is variable, and depending on its deformation, the central depressions are usually 250-300 nm according to section analysis. In the large scan area (Figs. 1a, 1b and 1c), the overall morphology of the cells is visible, but detailed structural features on the cell surface are not resolved. Off-line zooming into the above AFM images still cannot resolve the fine structure. Obviously, the AFM images obtained from these scans (larger than the diameter of the red blood cell) do not contain any more detailed structural information on the surface of red blood cell.

AFM images of the fine structure of partial small areas on the surface of red blood cells

As shown in Figure 2a, by zooming in on the surface of a red blood cell to a smaller scan size, 2.5 x 2.5 μm^2, the fine structure of the surface of the red blood cell can often be resolved. With a 2.5 x 2.5 μm^2 scan size, the smallest features on the erythrocytes that can be resolved are about 10 nm. Similarly, higher magnifications of AFM images can be achieved by progressive real-time zooming to scan sizes of 1.0 x 1.0 μm^2, 500 x 500 nm^2, and 200 x 200 nm^2 scan sizes (Figs. 2b, 2c and 2d, respectively), and the best resolution of the AFM image for the case of 200 x 200 nm^2 can reach about 2 nm. Hence, high resolution can be achieved on small scan areas. On one hand, high resolution AFM images with nanometer resolution require that the scan be performed on limited areas much smaller than the entire red blood cell, while on the other hand, if the scan area covers the entire red blood cell, the resolution of the AFM image decreases and detailed structures are not resolvable. It seems difficult at first sight to use AFM to image red blood cell with a large enough scan size to cover the entire surface and with nanometer resolution, simultaneously.

AFM image of the fine structures of the entire surface of a red blood cell

The key problem is how to visualize the surface structure of the whole red blood cell without loss of the high resolution of AFM. The first step was to search for an isolated and typical red blood cell with a smooth surface. Such a cell is seen in middle-left area of Figure 1a. The AFM image of the red blood cell is magnified as shown in Figure 1c, but without improvement in resolution. To map the whole surface of the red blood cell with nanometer resolution, the experiment was performed by dividing the cell surface into 28 sub-areas as shown in Figure 3, and then using a step-and-scan method. Each sub-area was 2.5 x 2.5 μm^2. The AFM tip was first moved to sub-area 1, which corresponded to the most bottom-right region of the cell in Figure 1c. Scan rate, proportional gain, and integral gain were adjusted to optimal values. Then, a TMAFM scan was

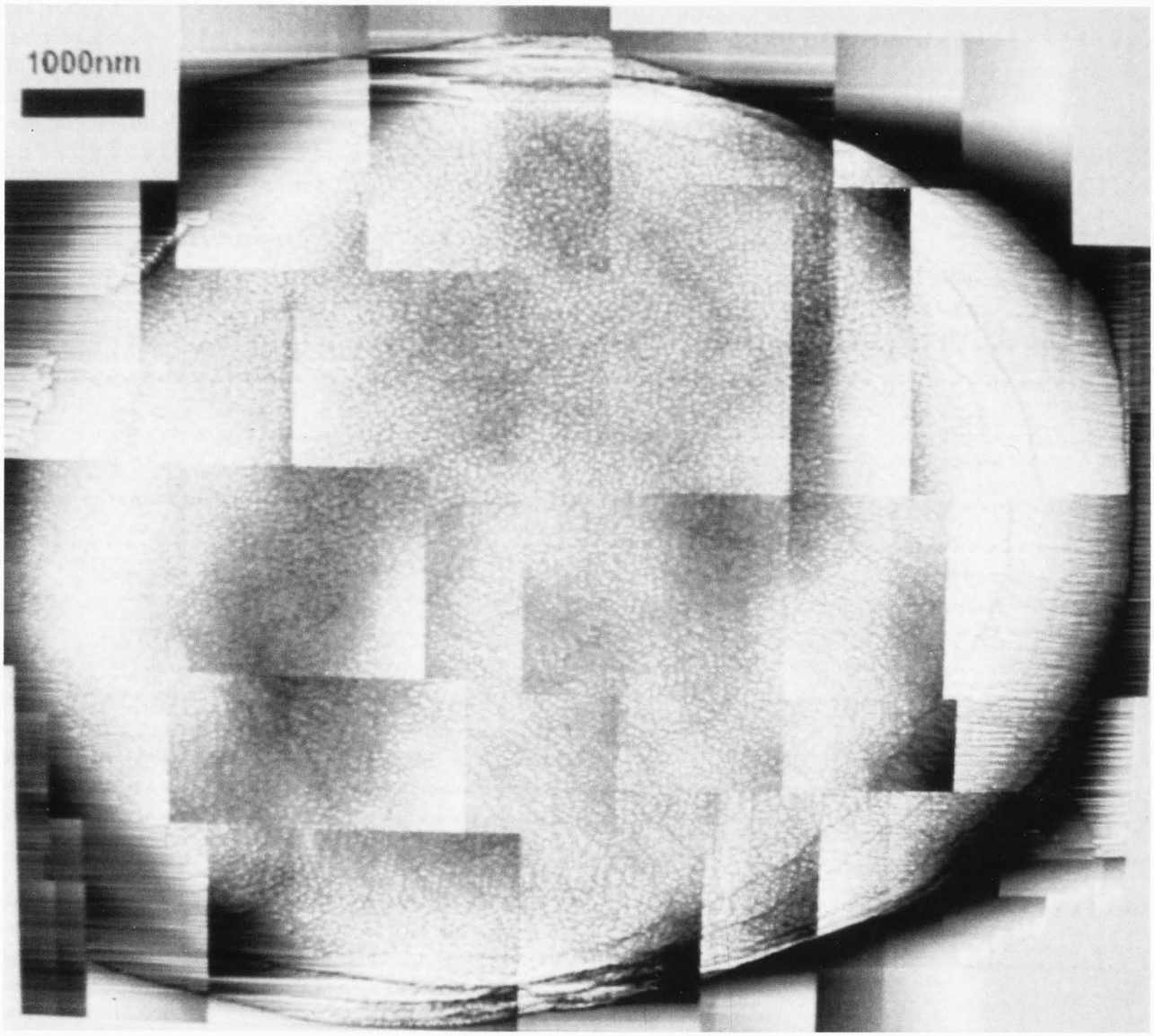

Figure 4. The AFM image of the entire surface of a red blood cell at nanometer-scale resolution. The whole image is constructed of 28 sub-area images with a scan size of 2.5 x 2.5 μm^2.

recorded. In most cases, if the scan rate is set to 1 Hz, and both proportional and integral gain adjusted to around 0.5, a good quality AFM image with nanometer resolution can be obtained. It is noted that for soft surfaces, such as surface of red blood cells, the scan rate should not be too fast in order to avoid distortion of the AFM image and sample damage. After sub-area 1 was imaged, the scan tip was moved 2.0 μm toward the left as shown by the arrow by changing the x-offset. In a similar way, high-resolution AFM imaging in sub-areas 2 to 5 was carried out. After the scan area had reached the most bottom-left position, it was moved 2 μm upward by changing Y-offset, so that sub-area 6 could be imaged. Similarly, sub-areas 7 to 28 were imaged in sequence along the direction indicated by arrows in Figure 3. All AFM images were acquired with the same parameters. It should be pointed out here that the scan size (2.5 x 2.5 μm^2) is always larger than tip movement between scans (2.0 μm), so each AFM image always overlaps with its neighbor images, even if thermal drift occurs. After all sub-areas were imaged, the images had to assembled to reveal the fine structure of the entire surface of the red blood cell. The detailed structural features of each AFM image have to be carefully identified, and its several adjacent sub-area images compared to identify actual overlaps. The AFM

image of the entire red blood cell was constructed in two steps. First, the AFM images of the sub-areas along the x-direction were combined using PBBRUSH software (Microsoft Windows Paintbrush, Version 3.1) to form partial structures based on each row. The partial structures of rows 1, 2, 3, 4 and 5 consist of sub-areas 1 to 5, sub-areas 6 to 11, sub-areas 12 to 17, sub-areas 18 to 23 and sub-areas 24 to 29, respectively. These row structures were then combined along the y-direction to give the morphology of the entire red blood cell with nanometer-scale resolution as shown in Figure 4.

Fine structural features of the surface of uncoated red blood cells

As can be seen in Figure 4, the most obvious characteristic of the uncoated external surface of the red blood cell is the presence of "particulate" components with a size ranging from a few nanometers to a few hundred nanometers. They exhibit variable shapes including globoid and ellipsoid ones, and are organized into closely-packed structures. There is no obvious difference in their distribution between the center and the periphery of the surface of the red blood cell. The boundaries of these particles can be identified clearly by zooming to higher magnification as shown in Figures 2b, 2c and 2d. Between particles, irregularly curved groove-like structures are visible. These grooves are a few nanometers wide and 2 nm deep. No jumping protrusion or depression structures were found on the entire surface. The height change of the surface from periphery to center is very smooth.

As is well-known, the lipid-globular protein mosaic model (LGPM) has been developed independently by Leonard and Singer (1966), Singer and Nicolson (1972) and Wallach and Zahler (1966). Singer and Nicolson (1971) introduced two terms to describe proteins associated with membrane lipid bilayers: integral proteins and peripheral proteins. They suggested that the integral proteins are amphipathic proteins with an ionic exterior segment in contact with water at the external surface of the membrane and a hydrophobic interior segment embedded in the lipid layers. Peripheral proteins are at the surface of the membrane, and do not intercalate with the lipid matrix of the membrane. Proteins might exist either singly or as subunit aggregates. Figure 4 shows two-dimensional surface structural features of the red blood cell. The AFM images provide the positions, shapes, and sizes of these particulate components over the entire surface of the red blood cell at a nanometer scale. In the high magnification image shown in Figure 2d, the spatial relationships among these particles are also revealed. The shape of some of particles in Figure 2d seems to be complementary in space. In the lateral dimension, there may also be mosaic structures among these particles. They are closely packed altogether, but the curved grooves or narrow gaps between the particles can also be identified in 500 x 500 nm^2 and 200 x 200 nm^2 scan sizes. The particulate components observed in Figures 2c and 2d may be associated with integral proteins and peripheral proteins in LGPM. Each of the particles from a few nanometers to about 20 nm wide may correspond to a single protein on the surface of the red blood cell. The larger particles (more than about 20 nm probably represent protein aggregates of several protein molecules. The fine structures of the entire uncoated surface of the red blood cell revealed by these AFM images, may form more direct and comprehensive evidence for the LGPM model.

Conclusions

As described above, TMAFM is a novel technique, which can reveal surface structures of red blood cells from a cellular down to nanometer level. It allows the observation of cell morphology at different magnifications, covering the ranges of both optical microscope and SEM. TMAFM has a higher resolution than the optical microscope, and sample preparation for TMAFM is more convenient than for SEM. The AFM image in Figure 4 provides integral information on the entire surface of the red blood cell at high resolution down to a few nanometers. The AFM images demonstrates that the uncoated red blood cell surface exhibits a characteristic structure composed of a large amount of close-packed particles with sizes ranging from a few nanometer to tens of nanometers. These particulate components are evenly distributed and no jumping protrusion or depression structures were found. These particles give rise to a very smooth surface of the red blood cell as seen in the large scan images. The closely-packed particle-like morphology may be closely associated with the two-dimensional distribution of isolated proteins or protein aggregates on the surface. The results obtained from the continuous recording of 28 AFM images for about 3 hours indicate that TMAFM can image soft biological samples such as red blood cell stably and reproducibly. AFM is a novel and convenient tool for directly observing fine structural changes related to biochemical processes such as aging and free-radical damage of red blood cells. The above results open a new door to investigate structure-function relationships of red blood cells at the nanometer scale.

Acknowledgments

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Discussion with Reviewers

J. Vesenka: The authors need to defend the use of a fixative and imaging in air when both clearly describe non-physiologically relevant conditions. They might also consider investigating tapping mode in buffer on live blood cells, currently available from the AFM manufacturer as a user-installed kit.

C. Le Grimellec: Glutaraldehyde is a cross-linker. There is also a high probability for air-drying induced changes in protein conformation. Accordingly, it seems hazardous to interpret the images in the way that the authors have done.

Authors: Glutaraldehyde is indeed a protein cross-linker. This substance has been extensively used in the fixation of specimens for electron microscopy, including proteins. Therefore, we also used the reagent in AFM sample preparation. On the other hand, preparation for electron microscopy usually requires drying in vacuum, which is more drastic than air-drying. This treatment may also induce changes in protein conformation. The information that we are attempting to extract from the AFM considers the morphology of the bio-macromolecules rather than their conformation. In fact, it is very

difficult to use the microscope directly to identify secondary structure such as α-helix, β-sheet, and turns, and to say nothing about their three-dimensional conformation. At present, if one wants to measure the three-dimensional conformation of a protein in solution, nuclear magnetic resonance (NMR) may be a more feasible method. Nevertheless, so far only for a few proteins (in solution) with low molecular weight, the three-dimensional conformation was determined by NMR. The exact conformation of many proteins in solutions is unknown. So how do we judge the difference in conformation between the protein in solution and after air-drying? Perhaps a reagent, that will preserve the protein conformation so that it is the same after drying compared to in solution, can be found.

C. Le Grimellec: The authors assume that they are using a very low force during tapping. Have they any idea of this force? Forces around 0.55, which is not very low, were recently reported for tapping under liquid (Putman *et al.*, Biophys. J. **67**: 1749-1753, 1994).
Authors: The low force mentioned in the paper means low lateral force achieved by tapping rather than vertical force. Putman *et al.* report on an exciting method about tapping in liquid so as to reduce the vertical interactive force.

C. Le Grimellec: What was the atmospheric humidity during these experiments?
Authors: The precise humidity during the experiments was not measured, but in view of the Beijing climate, the atmospheric humidity during the experiment is estimated to be very low.

H.J.K. Hörber: In none of the images, I see the claimed resolution of 2 nm. The smallest two-dimensional structure that I can identify is more than 20 nm, and microscopic resolution is defined in two dimensions, not, as often is done, on edges.
Authors: In the AFM image taken at 200 x 200 nm, the grooves between particles on the cell surface can be distinguished. The width of these grooves is about 2 nm.

E. de Harven: The reconstruction of Figure 4 is a futile technical exercise, since nobody would ever think of using the AFM for viewing whole cells.
Authors: The reconstruction of the red blood cells seems to be necessary, because so far there is not enough evidence to demonstrate that the structure of the entire red blood cell surface can be deduced from its partial structures. The local structural information does not mean the exact and thorough understanding of the entire red blood cell.

Reviewer VI: Lipids are an integral part of membranes. Why do the authors not mention lipids?
Authors: Lipid molecules are small and cannot be seen with our method. In addition, we have not yet found a method which can use the AFM to distinguish between proteins and lipids.

Reviewer VII: Any meaningful interpretation of surface features, particularly on aldehyde-fixed specimens, requires some type of positive identification of specific surface structures. Colloidal gold labeling is one such method of identification. Please comment.
Authors: Use of labeling methods to determine the location of specific components on the cell surface is indeed a good suggestion. However, the aim of the present study was to measure the total distribution of all components rather than that of a single component. We plan to utilize labeling methods in our future work.

E. de Harven: I wonder whether red blood cells are not the only cells one could study with the AFM because of their flat, structure-less surface. Any other blood cell would certainly demand a level of z contrast/imaging which is probably impossible to provide with AFM. In other words, your choice of RBC underlines the considerable restriction and limitations of the use of AFM in cell surface imaging. Please comment.
Authors: Red blood cells are not the only cells one could study with AFM. In principle, all cells including other blood cells can be imaged with AFM. When the surface roughness of a measured cell is much smaller than its height, gray-scale limited vertical resolution should be noted, in addition to pixel-limited lateral resolution. For example, a vertical change of less than 3.9 nm on the surface of a cell will not be detected for the height of the cell larger than 1000 nm and the image acquired in 256 gray-scales, if we scan only one image to measure the whole cell. Imaging the surface by dividing it into suitable sub-areas will help to improve image resolution. Of course, the resolution of AFM image also ultimately depends on sample surface characteristics. RBC is the first cells on which we have applied this AFM methodology to identify nanometer structures over a complete cell. Other cells with larger surface features make less demand on Z contrast.